36 Modelling pyruvate distribution in *Lactococcus lactis*: a kinetic model to support metabolic engineering strategies

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Introduction

Lactococcus lactis is used in starter cultures for the manufacture of various dairy products. The major end product of fermentation is lactate (> 95%). Various other end products are important for the flavour (e.g., diacetyl), but are only produced in minute amounts. Some applications require a considerably higher production of these minor products. Metabolic engineering is employed to divert the flux from lactate to these other end-products. For efficient metabolic engineering a thorough understanding of the metabolism is essential. More advanced is the construction of a kinetic model that can be used in combination with metabolic control analysis (MCA) to determine the most cost effective way to engineer the metabolic fluxes. Here we describe how a kinetic model was used successfully to modify the pyruvate metabolism of *L. lactis*.

[1]Abbreviations: ALS, acetolactate synthase; LDH, lactate dehydrogenase; NOX, NADH oxidase.

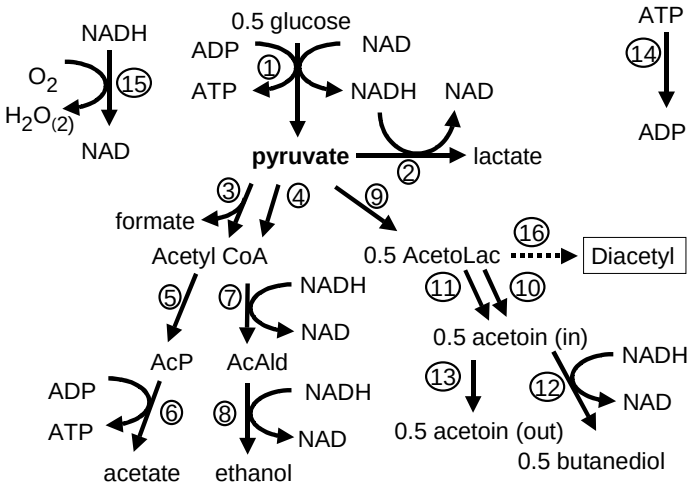

Fig. 36.1 Reactions included in the model to describe the distribution of carbon from pyruvate in *L. lactis*. Numbers in circles indicate the following enzymes or steps: 1 Lumped glycolysis; 2 Lactate dehydrogenase (LDH); 3 Pyruvate formate lyase; 4 Pyruvate dehydrogenase; 5 Phosphotransacetylase; 6 Acetate kinase; 7 Acetaldehyde dehydrogenase; 8 Alcohol dehydrogenase; 9 Acetolactate synthase (ALS); 10 Acetolactate decarboxylase; 11 Non-enzymatic Acetolactate decarboxylation; 12 Acetoin dehydrogenase; 13 Acetoin efflux; 14 ATP-ase; 15 NADH-oxidase (NOX); 16 Chemical conversion to diacetyl, not included in the model. Substrate and products that were clamped in the model are indicated in italics.

Methods

The model A kinetic model was generated to describe the conversion of glucose to the various end-products. The conversion of glucose to pyruvate was lumped to one step in which ATP and NADH are produced (Fig. 36.1). The reaction was described with irreversible Michaelis-Menten kinetics with product inhibition. ATP demand of the cell was represented by an ATP-ase with kinetics that ensured a tight regulation of the ATP/ADP ratio. For most other reactions kinetic parameter values were used as they have been published for *L. lactis*. Most reactions were described with reversible Michaelis-Menten kinetics. For all of these the V_{max} values for *L. lactis* have been determined and for most of these the substrate affinity constants have been published. Where the relevant *L. lactis* information was missing *E. coli* kinetics were used. Conversion of acetolacte to acetoin occurs both enzymatically (by acetolactate decarboxylase) and non-enzymatically. Non-enzymatic conversion of acetolactate to acetoin was described with mass action kinetics. The model contained three moieties: NAD, ADP, CoA with total sums of 10, 5 and 1 mM, respectively. Pathway substrate and products concentrations were clamped [1].

The model focuses on the flux through ALS (acetolactate synthase) because

this is the step converting pyruvate to acetolactate, the precursor for diacetyl, an important flavour component. However, only a very small part is converted to diacetyl and therefore the acetoin production is taken as a measure for the ALS flux.

Simulations and steady-state calculations of the kinetic model were performed with the metabolic modelling program SCAMP [2].

Experimental The metabolic fluxes in *L. lactis* were studied in batch cultures. The cells were inoculated in a stirred (500 rpm) fermenter containing 1 L. M17 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose. The pH was maintained at 6.0. Every 60 min the biomass (OD) and the concentration of glucose and products in the medium were determined. From these the fluxes in the exponential growth phase were calculated.

Metabolic engineering Lactate dehydrogenase (LDH) was inactivated in *L. lactis* MG5267 by disruption of the *ldh* gene by chromosomal integration [3]. The *als* gene was overproduced in *L. lactis* by using a multicopy vector, leading to a 100-fold over-expression of acetolactate synthase activity [3].

Results

The model predicted that in wild type 97% of the glucose will be converted to lactate, in agreement with our observations. Our interest was to increase the flux through ALS, that converts pyruvate into acetolactate, a precursor for diacetyl formation. MCA showed that the control of ALS on its own flux is close to 1 under anaerobic conditions and 0.64 under aerobic conditions. Often, when the aim is to increase the flux through a branch of a pathway, the first enzyme in that branch is over-expressed. The high control coefficient of ALS on its own flux seems to agree with such a strategy. However, MCA indicated that two other enzymes have a much higher control on the flux through ALS. First, LDH which has a very large negative control coefficient (−4.9) and secondly, NOX (NADH oxidase) which has a large positive control (1.6). This indicates that manipulation of these enzymes may be more efficient with respect to increasing the flux through ALS than over-expressing ALS.

The model predicted that a five-fold increase in ALS should increase the flux to acetoin to about 1% of the total glucose flux under aerobic conditions. Anaerobically, the flux through ALS should still be negligible. On the other hand, the model predicted that in the LDH negative mutant the flux through ALS should be 24% (anaerobically) and 97% (aerobically)of the glucose consumption flux.

To test these predictions *L lactis* mutants (an LDH knock-out and an ALS over-expressing mutant) were generated and their metabolic fluxes studied. In prelimi-

nary experiments the fluxes in the mutants were analysed. Aerobically the mutant over-expressing ALS showed an increase in the flux to acetoin, to about 7%. Anaerobically there was no significant flux through ALS, as we found for the wild type. The ALS flux in the LDH negative mutant was much higher, 62% of the glucose was converted to acetoin. Again the flux under anaerobic conditions was much lower (10%). The mutant where LDH disruption was combined with ALS over-expression gave a significantly higher flux to acetoin under anaerobic conditions (39%). Also aerobically the flux was increased compared with the LDH negative strain. The model predicted that disrupting LDH should decrease the carbon flux considerably. Our preliminary experimental results seem to confirm this and these strains still showed some residual LDH activity.

The other enzyme with a high control coefficient on the flux through ALS was NOX and the model predicted that a 150-fold over-expression would lead to a 33% conversion of glucose to acetoin. The effects of NOX over-expression have thus far only been studied in a system were the end products (and not the fluxes) of a fermentation were determined. In a strain over-expressing NOX, 57% of the lactose was converted to acetoin compared with 3% in the wild type, clearly demonstrating that this is an efficient way to redirect the carbon flux. The model predicts that NOX over-expression, unlike LDH disruption, should not lead to a decrease in the carbon flux.

Discussion

This study demonstrates that the combination of kinetic modelling with MCA is a powerful tool to guide metabolic engineering. The detailed kinetic model made it possible to predict the fluxes in mutants. The MCA indicated the potential targets for metabolic engineering and the kinetic model was used to predict the effect of the suggested modifications on the fluxes.

It is interesting to see that ALS was not the best target of metabolic engineering to increase the ALS flux, despite the fact that ALS had a control close to 1 on its own flux. This could be explained, as MCA revealed, by the much stronger control by two other enzymes. One of these was LDH which competes successfully with ALS for pyruvate at the normally low pyruvate (around 1 mM) concentrations, due to the very low affinity of ALS for pyruvate (K_m = 50 mM). The other was NOX which oxidises NADH without utilising carbon (for NADH oxidation by LDH or ADH also pyruvate is required). A highly active NOX should reduce the NADH/NAD ratio, which in turn should reduce the flux through LDH and at the same time increase the flux through glycolysis. As a result the cellular pyruvate concentration will increase and with this the flux through ALS should increase.

The model predictions showed that the increases in the ALS flux achieved by disrupting LDH or by over-expression of NOX cannot be approached by simply over-expressing ALS. This clearly demonstrates that the use of a kinetic model

in combination with MCA may be essential to reveal which enzymes have to be modified to redirect the flux. Moreover, it shows that the same results sometimes can not be achieved by simply over-expressing (all) the enzymes of the branch through which the flux has to be increased.

References

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